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(54) **Highly sensitive assay method for myo-inositol and composition for practicing same.**

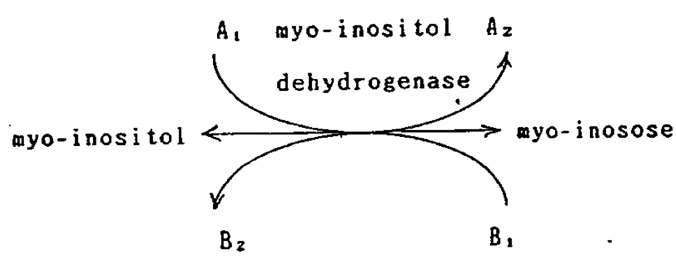
(57) A method of assaying a specimen for myo-inositol which comprises reacting the specimen with:

a) myo-inositol dehydrogenase using one of the thionictoinamide adenine dinucleotide phosphate group (thio-NADP group) or thionicotinamide adenine dinucleotide group (thio-NAD group) and one of the nicotinamide adenine dinucleotide phosphate group (NADP group) or nicotinamide adenine dinucleotide group (NAD group) as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction form myo-inosose from myo-inositol.

b) A_1, and

c) B_1;

to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

B_2 is an oxidized form of B_1; and

measuring the amount of A_2 or B_1 generated or consumed in the cycling reaction.

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This invention relates to a highly sensitive method of assaying a specimen for myo-inositol, to a composition suitable for assaying myo-inositol, and to a myo-inositol dehydrogenase and its production.

Myo-inositol is the one of nine isomers of inositol and is a stable cyclic alcohol. In humans myo-inositol is supplied in an amount of approximately one gram per day from daily meals and approximately 2 grams from renal biosynthesis.

The blood level of myo-inositol is controlled to a constant level by a balance of intake into cells and renal excretion, reabsorption and oxidation.

The plasma level of myo-inositol is increased by a renal functional disorder [Clinical Chem., 24(11); 1448-1455 (1988)]. The renal function can be monitored by measuring the level of myo-inositol in the blood.

Heretobefore the amount of myo-inositol in a specimen has been measured by gas-chromatography and high performance liquid chromatography (HPLC). These measuring methods have a number of disadvantages, for example the specimen has to be previously treated and the operation needs a complex treatment. Hence large numbers of assays for clinical chemistry was impossible. The normal standard level of myo-inositol in blood plasma is quite low, such as approximately 30 μmol/l [Proceedings of Japan Clinical Chemistry Annual Meeting No. 28 (1988)]. A simple and highly sensitive assay method is desired.

An enzymatic cycling method for assaying trace amount of substrates or enzyme activity using two types of enzyme to amplify the sensitivity is known. Among them, NAD-cycling, CoA-cycling and ATP-cycling are known but are almost never applied to routine assay methods for clinical chemistry due to complex operations.

We have found that myo-inositol dehydrogenase (EC. 1.1.1.18) can be used in a cycling reaction with thio-NADP group coenzymes.

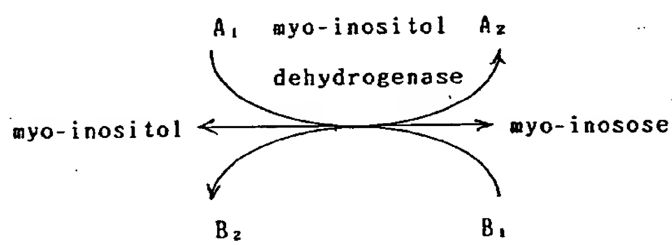
The present invention provides a method of assaying a specimen for myo-inositol which comprises reacting the specimen with:

a) myo-inositol dehydrogenase using one of the thionictoinamide adenine dinucleotide phosphate group (thio-NADP group) or thionicotinamide adenine dinucleotide group (thio-NAD group) and one of the nicotinamide adenine dinucleotide phosphate group (NADP group) or nicotinamide adenine dinucleotide group (NAD group) as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1, and

c) B_1;

to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

B_2 is an oxidized form of B_1; and

measuring the amount of A_2 or B_1 generated or consumed in the cycling reaction.

The present invention also provides a method of assaying a specimen for myo-inositol which comprises reacting the specimen with:

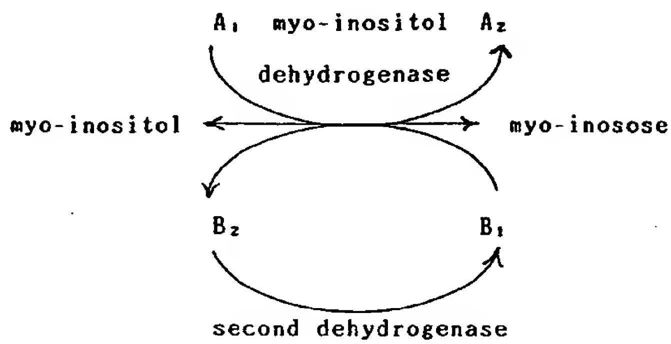
a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1,

c) B_1 or/and B_2, and

d) a second dehydrogenase which does not act on myo-inositol and which catalyses a reaction forming B_1

from B_2, and a substrate for the said second dehydrogenase;
to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

B_2 is an oxidized form of B_1;

the reaction from B_2 to B_1 being an enzymatic reaction which generates B_1 from B_2 by an action of the second dehydrogenase with a coenzyme; and

measuring the amount of A_2 generated in the cycling reaction.

The present invention further provides a method of assaying a specimen for myo-inositol which comprises reacting the specimen with:

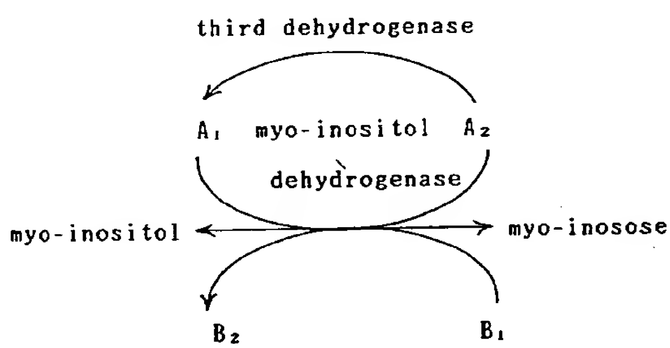
a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 or/and A_2,

c) B_1 and

d) a third dehydrogenase which does not act on myo-inositol and which catalyses a reaction from A_2 to A_1, and a substrate for the said third dehydrogenase;

to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a NADP or NAD group compound,

and

B_2 is an oxidized form of B_1;

the reaction from A_2 to A_1 being an enzymatic reaction which generates A_1 from A_2 by an action of the third dehydrogenase with a coenzyme; and

measuring the amount of B_1 consumed in the cycling reaction.

The present invention additionally provides a reagent composition suitable for assaying myo-inositol which comprises:

a) myo-inositol dehydrogenase using one of the thionicotinamide adenine dinucleotide phosphate (thio-NADP group) or thionicotinamide adenine dinucleotide group (thio-NAD group) and one of the nicotinamide adenine dinucleotide phosphate group (NADP group) or nicotinamide adenine dinucleotide group (NAD group) as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 and

c) B_1;

wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound.

The present invention also provides a reagent composition suitable for assaying myo-inositol which comprises:

a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1,

c) B_1 or/and B_2, and

d) a second dehydrogenase which does not act on myo-inositol and which catalyzes a reaction forming B_1 from B_2, and a substrate for the said second dehydrogenase,

wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

B_2 is an oxidized form of B_1.

The present invention further provides a reagent composition suitable for assaying myo-inositol which comprises:

a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 or/and A_2

c) B_1 and

d) a third dehydrogenase which does not act on myo-inositol and which catalyzes a reaction from A_2 to A_1 , and a substrate for the said third dehydrogenase;

wherein:

A_1 is thio-NADP, thio-NAD, NADP or NAD group compound,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

A_2 is an oxidized form of A_1.

The present invention additionally provides a myo-inositol dehydrogenase which has substrate specificity for myo-inositol and which catalyses the reaction:

$$\text{myo-inositol} + \text{NAD} \rightleftharpoons \text{myo-inosose} + \text{reduced NADH}$$

The present invention also provides a process for the production of a myo-inositol dehydrogenase which comprises culturing a myo-inositol dehydrogenase producing microorganism belonging to genus Bacillus, and isolating myo-inositol dehydrogenase from the cultured mass.

The present invention further provides Bacillus sp. No. 3 (FERM BP-3013) or a mutant thereof which is

capable of producing a myo-inositol dehydrogenase.

The present invention additionally provides a culture of Bacillus sp. No. 3 (FERM BP-3013), or a mutant thereof which is capable of producing a myo-inositol dehydrogenase, in a culture medium which comprises a source of assimilable carbon, a source of assimilable nitrogen and mineral salts and which is free of other microorganisms.

The assay method of the present invention depends on the difference in the maximum absorption of a reduced thio-NAD(P) group, which is an analogue of NAD(P), at approximately 400 nm, and that of a reduced NAD(P) group at approximately 340 nm,. In the enzymatic cycling reaction using myo-inositol dehydrogenase, a thio-NAD(P) group is used as one of the two coenzymes, and the change in amount of any of the coenzymes is measured, for example by the difference in maximum absorptia of both reduced coenzymes. Thus the amount of myo-inositol in a specimen can be most precisely measured.

Brief Description of the Drawings

Figure 1 : heat stability of the novel myo-inositol dehydrogenase
Figure 2 : optimum temperature of the myo-inositol dehydrogenase
Figure 3 : pH-stability of the myo-inositol dehydrogenase
Figure 4 : optimum pH of the myo-inositol dehydrogenase
Figures 5 - 8 : assay curve of myo-inositol.

Deatiled Description of the Invention

In the present invention, any type of myo-inositol dehydrogenase can be used that has the properties hereinabove. Examples of myo-inositol dehydrogenase suitable for use in the present invention are described in Enzyme Handbook (Asakura Publishing Co., Tokyo) and are myo-inositol dehydrogenase produced by the following microorganisms.

Aerobacter aerogenes [J. Biol. Chem., 241, 800-806 (1966)] : Klebsiella pneumoniae, Seiratia marcescens, Cryptococcus melibiosum [Biochim. Biophys. Acta.,293, 295-303 (1973)] : and Bovine brain (Biochem. Biophys. Res. Comm.,68, 1133-1138 (1976)] : Bacillus sp. No. 3 (product of Toyo Jozo Co.).

Among these, Aerobacter aerogenes, Klebsiella pneumoniae and Serratia marcescens are known as etiologic microorganisms for pneumoniae and opportumistic infections (Standard Microbiology., p. 209-212, Igaku Shoin Publ., Tokyo) with inveterate infection resistant to chemotherapeutics and antibiotics. Culturing these microorganisms in on industrial scale is substantially impossible. Km-value of myo-inositol dehydrogenase produced by yeast Cryptococcus melibiosum is approx. 11.0 mM for myo-inositol and approx. 0.07 mM for NAD, which are so high value of Km to get sufficient reaction rate. (Enzyme Handbook, p. 6)

We have made screening search for obtaining microorganisms which can produce myo-inositol dehydrogenase, without non-infectious, higher activity, low Km-value on substrate myo-inositol and NAD, stableand easily purify and isolated a microorganism designated Bacillus sp. No. 3 from a soil sample in a hot spring area, Atagawa, Higashi-Izu-cho, Kamo-gun,Shizuoka-ken, Japan.

Myo-inositol dehydrogenase produced by Bacillus sp. No. 3 has Km-values for myo-inositol and NAD at pH 8.5 are quite low values of approx. 0.6 mM and 0.04 mM, respectively, with high reactivity. Moreover remaining activity after treating at 60°C for 15 minutes in the buffer solution is kept over approx. 95 %. The enzyme, myo-inositol dehydrogeanse is a novel enzyme which can act with coenzyme NAD(P) group and thio-NAD(P) group.

The said novel enzyme myo-inositol dehydrogenase can also be produced by myo-inositol dehydrogenase producing microorganism belonging to genus Bacillus and isolating the enzyme therefrom.

The present myo-inositol producing microorganism belongs genus Bacillus, and Bacillus sp. No. 3 which has isolated by the present inventors is the most preferable producing microorganism, however the said microorganism is to be understood as an example.

The taxonomical properties of this strain are as follows:

(a) Morphological properties:

Round edge with straight or slightly curved bacillus. Sizes are 0.5 ~ 07 x 1,5 ~ 3.5 μ m. Peritrical movement. Spore formations in an edge or subedge with sizes in 0.8 x 1.0 ~ 2.0 μ m of elliptical to oval spores are observed. Microbial cells are swollen with spores. No polimorphism.

(b) Growth on various media:

Observations on various media, cultured for 1-2 days at 50-52°C, are as follows:
1. Nutrient agar plate medium : round and convex colonies. Smooth wettish surface with round edge. Ocherous or plate ocherous. No formation of soluble pigment.
2. Nutrient agar slant medium : good growth with filiform. Ocherous to plate ocherous. No formation of soluble pigment.
3. Liquid medium (aqueous peptone) : good growth with uniform turbidity.
4. Litmus milk medium : weakly acidic after 4-5 days.
GC mole % in DNA : 41.9 mole % (HPLC method)
Main isoprenoid quinone : MK - 7

(c) Physiological properties (+ = positive, (+)=weakly positive, = negative, NT= not tested)

Gram-strain		+
KOH reaction		−
Capsule formation		−
Acid fastness stain		−
OF-test (High Leifson)		N.T
OF-test (nitrogen source: $NH_4H_2PO_4$)		F
Aeroboc growth		+
Anaerobic growth		+
Growth temperature	70 ℃	−
	60 ℃	+
	37 ℃	+
	30 ℃	−
Halotolerant NaCl conc.	%	
	0 %	+
	3 %	+
	5 %	−
Growth pH		

	pH 5.6	−
	pH 6.2	+
	pH 9.0	+
Gelatin hydrolysis		−
Starch hydrolysis		(+)
Casein hydrolysis		−
Esculin hydrolysis		+
Cellulose hydrolysis		−
Tyrosine hydrolysis		−
Arginine hydrolysis		−
Catalase production		+
Oxidase production		+
Lecithinase production		−
Urease production (SSR)		−
Urease production (Chris)		−
Indol production		−
H_2S production (detection: lead acetate paper)		−
Acetoin production (K_2HPO_4)		−
Acetoin production (NaCl)		−
MR test		−
Nitrate reduction		
	Gas detection	−
	NO_2^-	−
	NO_3^-	+

Utilization of Simmons medium	
Citrate	−
Malate	−
Maleate	−
Malonate	−
Propionate	−
Gluconate	−
Succinate	−
Utilization of Christenssen medium	
Citrate	+
Malate	−
Maleate	−
Malonate	−
Propionate	+
Gluconate	−
Succinate	−
Gas production from glucose	−
Acid formation from sugar	
Adonitol	−
L (+) arabinose	−
Cellobiose	+
Dulsitol	−
Meso-erythritol	−
Fructose	+
Fucose	+
Galactose	+

Glucose	+
Glycerin	+
Inositol	+
Inulin	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
Melezitose	−
Melibiose	+
Raffinose	−
Rhamnose	+
D-ribose	+
Salicin	+
L-sorbose	−
Sorbitol	−
Starch	+
Saccharose	+
Xylose	−
Trehalose	+

According to the above taxonomical properties, the microorganism displays the specific characteristics of Gram positive bacillus, namely, it is 0.5 ~ 0.7 x 1.5 ~ 3.5 μ m in size, is peritrichal in movement, is spore formation with no polymorphism, promotes fermentative decomposition of glucose and acid formation, is catalase positive and oxidase positive, and is thermophilic and facultative anaerobic.

Among Gram-positive bacillus with specific properties of spore formation and aerobic growth, the strain belongs to be genus Bacillus.

According to Bergey's Mannual of Systematic Bacteriology, Vol. 2, there are illustrated the following 9 species of Bacillus with growth in high temperature (50°C).

Bacillus acidocaldarius, B. subtilis, B. badius,
B. brevis, B. coagulans, B. licheniformis,
B. pantothenticus, B. schegelli and
B. stearothermophilus.

Among these, microorganisms growing at anaerobic condition are B. coagulance and B. licheniformis.

Taxonomic properties of Bacillus in comparison with those of the present strain, according to Bergey's Mannual, are illustrated by comparing Bacillus coagulans (hereinafter designated C) and Bacillus licheniformis (hereinafter designated L), as follows:

+ = positive
(+) = weakly positive

- = negative
d = not identified as + or -
ND = no data

		C	L	The present strain
Oxidase production		−	d	+
Swelling with spore		d	−	+
Anaerobic growth		+	+	+
Acetoin production		+	+	−
Glucose (acid)		+	+	+
L-arabinose (acid)		+	+	+
Xyrose		d	+	−
Mannitol		d	+	+
Casein hydrolysis		d	+	−
Gelatin hydrolysis		d	+	−
Starch hydrolysis		−	+	(+)
Citrate utilization		+	+	−
Propionate utilization		d	+	−
Tyrosine hydrolysis		−	+	−
LV-reaction		−	+	−
Indol production		−	+	−
Halotolerant	2 %	+	+	+
	5 %	−	+	−
	7 %	−	+	±
	10 %	−	ND	−
Growth trtemperature				
	40 ℃	+	+	+
	50 ℃	+	+	+
	55 ℃	+	+	+
	60 ℃	ND	ND	+

70 ℃	−	−	−
Nitrate reduction	d	+	−
GC mole % in DNA	44.5 (Type) 44.3 ∼50.3	46.4 (Type) 42.9 ∼49.9	41.9

According to the above comparison, the present strain No. 3 has many identical properties with Bacillus coagulans but has specific differences as to acetoin production and GC mole % in DNA. Further an observation on Litmus milk medium is different. (not mentioned in the above comparison).

Accordingly, the present strain has been designated Bacillus sp. No. 3 and has been deposited at the Fermentation Research Institute and assigned deposite No. FERM BP-3013.

In the present invention for production of the enzyme, myo-inositol dehydrogenase producing microorganism belonging to genus Bacillus is cultured in suitable medium.

Preferable example of the above myo-inositol dehydrogenase producing microorganism strain is Bacillus sp. No.3. Since taxonomical properties of bacterial are in general easily mutated, natural variants or artificial mutant produced by conventional artificial mutation treatment for example, ultraviolet irradiation, radiation or mutagen such as N-methyl-N-nitro-N-nitrosoguanidine and ethyl methansulfonate, within the scope of belonging genus Bacillus and having myo-inositol dehydrogenase producing activity, can be used in the present invention.

Culture the above microorganism can be performed by the conventional culture conditions used in bacteria. In the present enzyme production, since myo-inositol dehydrogenase is an inducible enzyme by myo-inositol, culture is preferably proceeded in a medium containing myo-inositol 0.5 ∼ 5 % to produce myo-inositol dehydrogenase with 10 ∼ 300 times productivity.

Among the examples of medium other than myo-inositol, a nutrient medium containing assimirable carbon source, digestible nitrogen source and if required inorganic salts can be used.

Example of carbon sources is glucose, fructose or saccharose which is used in combination or alone. Example of digestible nitrogen sources is peptone, meat extract or yeast extract which is used combination or alone. Further according to the requirement, phosphate, magnesium salt, calcium salt, potassium salt, sodium salt or various heavy metal salt such as ferric salt or manganese salt can be added. Other known assimirable carbon sources and digestible nitrogen salt can also be used.

Culture can be made generally shake culture or aeration culture with agitation, and in an industrial production, submerged aeration culture with agitation is preferable. Culture temperature can be altered within a range for production of the enzyme and is generally 40 ∼ 60°C, preferably approx. 50°C.

Culture time depends on the condition of culture, and can be set up for maximum production of the enzyme, and is generally 1 ∼ 2 days.

These composition of medium, medium condition, culture temperature, agitation speed and air flow should naturally be adjusted to preferable setting up the condition. Anti-foaming agent such as silicon oiland vegitable oil can also be used if required.

Since the thus produced myo-inositol dehydrogenase exixts as end enzyme in bacterial cells, cultured cells are collected by means of filtration or centrifugation, and the bacterial cells are decomposed by means of mechanical destruction such as ultra sonication, french press treatment, glass beads treatment or freezing-thawing, or enzymatic digestion such as lysozyme treatment, to obtain crude extract.

Further purified myo-inositol dehydrogenase can be obtained by known conventional purification method of isolation of protein or enzyme. For example, the enzyme can be precipitated by sulting-out method with adding ammonium sulfate or sodium salfate to the crude extract. The precipitated enzyme can further bepurified by means of combination of chromatography using molecular sieve and resin, electrophoresis or ultra-centrifugation.

The purification can be achieved by considering with nature of myo-inositol dehydrogenase. For example, the above precipitae dissolved in water or buffer solution is dialysed, if required, by semi-permeable membrane, and treated with chromatography using ion-exchange resin such as DEAE-cellulose, DEAE-Sephacel, DEAE-

Sepharose, DEAE-Sephadex, Q-Sepharose (Pharmacia Corp., trade name), DEAE-Toyoperal (Toso Corp.) or hydroxy apatite, hydrophobic chromatographic resin such as octyl -Sepharose or phenyl-Sepharose (Pharmacia Corp.) and other affinity chromatographic resin. Further molecular sieve chromatography using gel-filtration agent such as Sephadex G-100 or Sephacryl S-200 can be applied. Ifrequired desalting by means of semi-premeable membrane can also be applied. Purified myo-inositol dehydrogenase powder preparation can be prepared by lyophilization with addition of 0.05 ~ 10 % of stabilizing agent for example sugars such as mannitol, saccharose or sorbitol, amino acids such as glutamate or glycine, and peptide or such as bovine albumin.

Myo-inositol dehydrogenase of the present invention has the following properties.

1. Substrate specificity:

myo-inositol	100 %
glucose	0
fructose	0
galactose	0
sorbitol	0
mannose	0
maltose	0
saccharose	0
lactose	0

2. Enzyme action:

The enzyme catalyzes essentially a reaction of myo-inositol and NAD to generate myo-inosose and reduced NADH, as shown below.

$$\text{myo-inositol} + \text{NAD} \rightleftharpoons \text{myo-inosose}^{*} + \text{reduced NADH}$$

*(2, 4, 6/3, 5-pentahydroxy cyclohexanone)

3. Molecular weight:

130,000 ± 15,000

Measure by TSK-gel G 3000 SW 8 (Toso Co., 0.75 x 60 cm).

Elution : 0.1 M phosphate buffer (pH 7.0) containing 0.2 M NaCl.

Standard : following molecular markers (Oriental yeast Co) are used.

M. W. 12,400	Cytochrome C
M. W. 32,000	adenylate kinase
M. W. 67,000	bovine albumin
M. W. 142,000	lactate dehydrogenase
M. W. 290,000	glutamate dehydrogenase

4. Isoelectric point:

pH 4.5 ± 0.5

Measured by elelctrofocussing using carrier ampholite at 4°C, 700 V, for 40 hours. The activity of a fraction of each enzyme is measured.

5. Km-value:

0.64 mM (myo-inositol), 0.004 mM (NAD)

Km-value for myo-inositol is measured in various concentarations of myo-inositol in a reaction mixture of:

100 mM Tris-HCl buffer (pH 8.5)
5 U diaphorase (Toyo Jozo Co.)
1 mM NAD (Oriental yeast Co.)
0.025 % NBT (Wako Pure Chem. Co.)

In the reaction mixture, NAD is replaced by 15 mM myo-inositol, and the concentration of NAD is varied to measure the Km-value for NAD.

The results are as shown above.

Further in the reaction mixture, 1 mM NAD is replaced by 1 mM thio-NAD (Sigma Co.) and the Km-value for myo-inositol is measured. The result is as shown below.

Km-value: 10.0 mM (myo-inositol)

In the reaction mixture, 1 mM thio-NAD is replaced by 150 mM myo-inositol, and the Km-value for thio-NAD is measured.

Km-value :0.17 mM (thio-NAD)

Km-value in the reaction of NADP and myo-inositol are measured. The results are as shown below.

Km-value :0.19 mM (NADP)

Km-value :30.91 mM (myo-inositol)

Km-values in the reaction of the thio-NADP and myo-inositol are as shown below.

Km-value : 2. 54 mM (thio-NADP)

Km-value : 179. 62 mM (myo-inositol)

As clearly known hereinabove, the present enzyme can be reacted with using NAD(P) and thio-NAD(P) as the coenzymes.

6. Optimum pH:

In assay method for enzyme activity as illustrated hereinafter, 100 mM Tris-HCl buffer (pH 8.5) in the reaction mixture is replaced by 100 mM phosphate buffer (pH 6.5-8.0, -○-), 100 mM Tris-HCl buffer (pH 8.9-9.0, -□ -) and 100 mM glycine-NaOH buffer (pH 9.0-10.0,- ■ -), and incubated. The result is shown in Fig. 4.

A maximum activity is observed at approx. pH 9.5.

7. pH-stability:

The residual activity of the enzyme (1 U/ml, 40 mM buffer solution) is measured in various buffer solutions, i. e. acetate buffer (pH 4.5-6.0, - ▲ -) ; phosphate buffer (pH 6.0-8.0, -○-) ; Tris-HCl buffer (pH 8.0-9.5, - □ -) and glycine-NaOH buffer (pH 9.0-10, - ■ -) after heating at 50°C for 15 mins. The enzyme is stable at pH 6.5-9.0 with a residual activity of over 80 %, as shown in Fig.3.

8. heat-stability

The enzyme, dissolved in 20 mM Tris-HCl buffer (pH 7.0), to produce a 1 U/ml solution, is incubated for 15 min. at various temperatures, and the residual activity is measured.

The results are as shown in Fig. 1 and the enzyme is stable up to 60°C with maintaining residual activity of over 95 %.

9. Optimum temperature:

The enzyme activity is measured at 35, 40, 50, 55, 60 and 65°C, respectively, in 100 mM Tris-HCl buffer (pH 8.5) according to the assay method illustarted hereinafter. The reaction was stopped in each case after 10 mins. incubation by adding 0.1 N HCl (2 ml), whereupon the optical absorption was measured at 550nm. The enzyme shows maximum activity at 60°C as shown in Fig. 2.

10. Assay method of myo-inositol dehydrogenase

(1) Reaction mixture:

100 mM Tris-HCl buffer (pH 8.5)
15 mM Myo-inositol (Wako Pure Chem. Co)
1 mM NAD (Oriental Yeast Co.)
5 U Diaphorase (Toyo Jozo Co.)
0.025 % NBT (Wako Pure Chem. Co.)

(2) Enzyme Assay:

The above reaction mixture (1 ml) is incubated in a small test tube at 37°C for 5 mins. Diluted enzyme solution (0.02 ml) is added and stirred to initiate the reaction. After exactly 10 mins., 0.1 N HCl (2.0 ml) was added and stirred to stop the reaction. Absorption at 550 nm (A_{550} nm) is measured to obatain absorption A_1 . The assay was repeated using the above reaction mixture except that myo-inositol was not included. The mixture is also treated in the same manner as described above and its absorption Ao was measured.

(3) Calculation of enzyme activity:

$$U/ml = \frac{(A_1 - Ao)}{18.3} \times \frac{1}{10} \times \frac{3.02}{0.02} \times Z$$

wherein
18.3 : molecular absorption coefficient (cm^2/μ mol)
Z : dilution factor

In the enzymatic reaction hereinbefore illustrated, A_1 or B_2 is of the thio-NADP-group, thio-NAD group, NADP group or NAD group of coenzyme. Examples of the thio-NADP group or thio-NAD group are thionicotinamide adenine dinucleotide phosphate (thio-NAD) and thionicotinamide hypoxanthine dinucleotide phosphate or thionicotinamide adenine dinucleotide (thio-NAD) and thionicotinamide hypoxanthine dinucleotide. Examples of the NADP group or NAD group are nicotinamide adenine dinucleotide phosphate (NADP), acetylpyridine adenine dinucleotide phosphate (acetyl NADP) or nicotinamide hypoxanthine dinucleotide phosphate (deamino NADP) and nicotinamide adenine dinucleotide (NAD), acetylpyridine adenine dinucleotide (acetyl NAD) and nicotinamide hypoxanthine dinucleotide (deamino NAD).

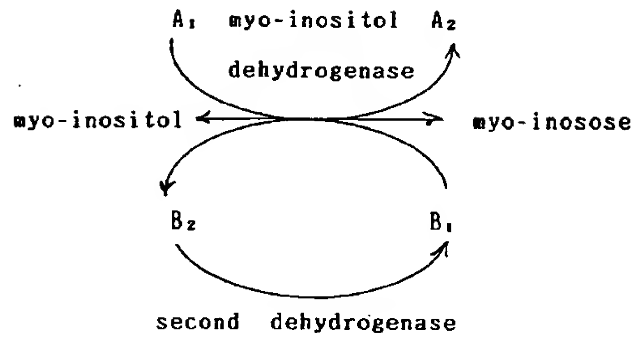
In the present invention, when A_1 is of the thio-NAD(P) group, B_1 is of the NAD(P)H group, and when A_1 is of the NAD(P) group, B_1 is of the thio-NAD(P)H group. Hence at least one will be a thio-type coenzyme.

Further when thio-NAD group and NAD group are coenzyme of myo-inositol dehydrogenase, any of thio-NAD group and NAD group hereinabove illustrated can be selected. Furthermore, when thio-NAD(P) group and NAD(P) group are coenzyme of the enzyme, any of thio-NAD group or thio-NADP group and NAD group or NADP group can be selected.

In a composition for an assay of myo-inositol according to the present invention, the concentration of A_1 and B_1 is 0.02-100 mM, preferably 0.05-20 mM, and the concentration of myo-inositol dehydrogenase is 5-1000 U/ml, preferably 2-500 U/ml. The amount exceeding the above range is acceptable.

A_1 and B_1 are used in excess as compared with myo-inositol and are in excess as compared with the Km-value (mM) of myo-inositol dehydrogenase for A_1 and B_1. Specifically a 20-10,000 times molar excess relative to myo-inositol is preferred.

In the present invention, 4th component of a second dehydrogenase which does not act on myo-inositol and which constitutes a reaction from B_2 to B_1 , and a substrate for the said second dehydrogeanse are combined to effect a cycling reaction of the formula (II) in which a reaction for regenerating the B_1 is added to the reaction $B_1 \rightarrow B_2$, and an amount of A_2 generated is measured.



(II)

wherein A_1 is thio-NADP group, thio-NAD group NADP group or NAD group, A_2 is a reduced form of A_1, when A_1 is thio-NADP group or thio-NAD group, B_1 is reduced NADP group or reduced NAD group and when A_1 is NADP groupor NAD group, B_1 is reduced thio-NADP group or reduced thio-NAD group, and wherein B_2 is an oxidized form of B_1, and the reaction from B_2 to B_1 is an enzymatic reaction which regenerate B_1 by an action of the second dehydrogenase with coenzyme of B_2. Namely the second dehydrogenase is added supplementary for regenerating the B_1 , thus an amount of B_1 can be reduced. B_1 can be replaced by B_2 or a mixture of B_1 and B_2 . In this case an amount of B_1 or/and B_2 is not limited, but is generally below 1/10 mole as of A_1, preferably 1/50 ~ 1/1000 mole, or less.

In a composition for an assay of myo-inositol with using the component (4) according to the present invention, the concentration of A_1 is 0.02 ~ 100 mM, preferably 0.05 ~ 20 mM, the concnetration of B_2 or/and B_1 is 0.05 ~ 5000 μ M, preferably 5 ~ 500 μ M, and the concentration of myo-inositol dehydrogenase is 5 ~ 1000 U/ml, preferably 20 ~ 500 U/ml. The concnetration of the second dehydrogenase is set up 20 times (U/ml) or more as compared with the Km-value of the second myo-inositol dehydrogenase for B_2 , for example preferably 1 ~ 100 U/ml. Substrate for the second dehydrogenase is used in excess, preferably 0.05 ~ 20 mM or more.

Examples of the second dehydrogenase and a substrate for the second dehydrogenase are as follows.

B_2 : NAD group or thio-NAD group;

Alcohol dehydrogenase (EC.1.1.1.1) and ethanol,

Glycerol dehydrogenase (EC.1.1.1.6)(E. coli) and glycerol,

Glycerol-3-phosphate dehydrogenase (EC.1.1.1.8) (rabbit muscle) and L-glycerol-3-phosphate,

Maleic dehydrogenase (EC.1.1.1.37)(porcine heart muscle, bovine heart muscle) and L-malate, and

Glyceraldehyde phosphate dehydrogenase (EC.1.2.1.12) (rabbit muscle, liver, yeast, E. coli) and D-glyceraldehyde phosphate and phosphate.

B_2 : NADP group or thio-NADP group;

Glucose-6-phosphate dehydrogenase (EC.1.1.1.49) (yeast) and glucose-6-phosphate,

Isocitrate dehydrogenase (EC.1.1.1.42)(yeast, porcine heart muscle) and isocitrate,

Glyoxylate dehydrogenase (EC.1.2.1.17) (Pseudomonas oxalaticus) and CoA and glyoxylate,

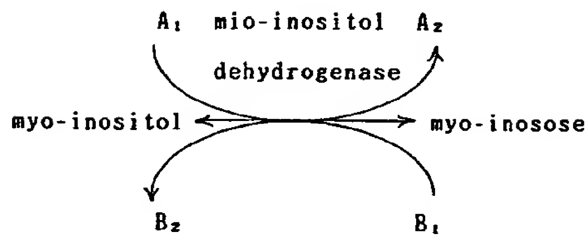
Phosphogluconate dehydrogenase (EC.1.1.1.44)(rat liver, beer yeast, E. coli) and 6-phosphogluconate,

Glyceraldehyde dehydrogenase (EC.1.2.1.13) (chlorophyll) and D-glyceraldehyde-3-phosphate and phosphate, and

Benzaldehyde dehydrogenase (EC.1.2.1.7)(Pseudomonas fluorescens) and benzaldehyde.

In the present invention, further 5th component of a third dehydrogenase which does not act on myo-inositol and which constitutes a reaction from A_2 to A_1 , and a substrate for the said third dehydrogenase are combined to effect a cycling reaction of the formula (III) in which a reaction for regenerating the A_1 is added to the reaction A_1 A_2 , and an amount of B_1 decreased is measured.

a third dehydrogenase



(III)

wherein A_1 is thio-NADP group, thio-NAD group NADP group or NAD group, A_2 is a reduced form of A_1, when A_1 is thio-NADP group or thio-NAD group, B_1 is reduced NADP group or reduced NAD group and when A_1 is NADP group or NAD group, B_1 is reduced thio-NADP group or reduced thio-NAD group, and wherein B_2 is an oxidized form of B_1 , and the reaction from A_2 to A_1 is an enzymatic reaction which regenerate A_1 by an action of the third dehydrogenase with coenzyme of A_2 . Namely the third dehydrogenase is added supplementary for regenerating the A_1 , and an maount of A_1 can be reduced. A_1 can be replaced by A_2 or a mixture of A_1 and A_2 . In this case an amount of A_1 or/and A_2 is not limited, but is generally below 1/10 mole as of B_1 , preferably 1/50 ~ 1/1000 mole, or less.

In a composition for an assay of myo-inositol with using the component (5) according to the present invention, the concentration of B_1 is 0.02 ~ 100 mM, preferably 0.05 ~ 20 mM, the concentration of A_2 or/and A_1 is 0.05 ~ 5000 μ M, preferably 5 ~ 500 μ M, and the concentration of myo-inositol dehydrogenase is 5 ~ 1000 U/ml, preferably 20 ~ 500 U/ml. The concentration of the third dehydrogenase is set up 20 times (U/ml) or more as compared with the Km-value of the third myo-inositol dehydrogenase for A_2 , for example preferably 1~ 100 U/ml. Substrate for the third dehydrogenase is used in excess, preferably 0.05 ~ 20 mM or more.

Examples of the third dehydrogenase and a substrate for the third dehydrogenase are as follows.

A_1 : NAD group or thio-NAD group;

Alcohol dehydrogenase (EC.1.1.1.1) and acetoaldehyde,

Glycerol dehydrogenase (EC.1.1.1.6)(E. coli) and dihydroxyacetone

Glycerol-3-phosphate dehydrogenase (EC.1.1.1.8) (rabbit muscle) and dihydroxyacetone phosphate,

Maleic dehydrogenase (EC.1.1.1.37)(porcine heart muscle, bovine heart muscle) and oxaloacetate and

Glyceraldehyde phosphate dehydrogenase (EC.1.2.1.12) (rabbit muscle, liver, yeast, E. coli) and 1, 3 -diphospho-D-glycerate.

A_1 : NADP group or thio-NADP group;

Glucose-6-phosphate dehydrogenase (EC.1.1.1.49) (yeast) and gluconolactone-6-phosphate, and

Glyceraldehude phosphate dehydrogenase (EC.1.2.1.13) (chlorophyll) and 1, 3-diphospho-D-glycerate.

The myo-inositol dehydrogenase used in the composition for assay of myo-inositol according to the present invention can be an enzyme having reactivity on a substrate of myo-inositol together with a suitable coenzyme NAD group, preferably NAD or thio-NAD group, preferably thio-NAD and NADP group, preferably NADP or thio-NADP group preferably thio-NADP. Its suitability can be confirmed by using the said coenzyme and substrate.

In the composition of the reaction medium, two coenzymes are selected by considering the relative activity of myo-inositol dehydrogenase on each coenzyme. Thereafter, the pH condition thereof on each optimum pH of the forward reaction and reverse reaction is adjusted to set up the pH-condition wherein a ratio of reaction rate on the forward reaction and reverse reaction approaches 1.

Myo-inositol dehydrogenase produced by Bacillus sp. No. 3 (product of Toyo Jozo Co.) has a relative activity of approx. 10 ~ 15 % when coenzyme thio-NAD is used, as compared to use of NAD. Optimum pH is approx. 9.5 for forward reaction and approx. 7 ~ 7.5 for reverse reaction. The enzyme can utilize both NAD group and NADP group as coenzyme.

In the present invention, myo-inositol dehydrogenase from a single origin or from plural origins can be used.

Myo-inositol in a specimen can be assayed by adding 0.001 ~ 0.5 ml of a specimen to the assay composition containing the above components (1) - (3), components (1) - (4) or components (1) - (3) and (5), reacting

at approx. 37°C, then measuring an amount of generated A_2 or consumed B_1 over an interval spanning two time points after starting the reaction, for example a minute between 3 mins. and 4 mins. after starting, or five minutes between 3 mins. and 8 mins. after starting the reaction. Measurement is effected by determining the changes of absorption at each optical absorption. For example, when A_2 is thio-NADH and B_1 is NADH, generated A_2 is measured by an increase of absorption at 400 nm or consumed B_1 is measured by a decrease of absorption at 340 nm and the thus-obtained value is compared with the value of a known concentration of reference myo-inositol, whereby a concentration of myo-inositol in a specimen can be measured in real time.

According to the assay method of the present invention, since myo-inositol itself existing in a specimen is introduced into the enzymatic cycling reaction, it is little affected by any coexisting substances in the specimens, and hence a measurement of a blank value of the specimen is not required. Thus, a simple assay system using a rate assay can be achieved.

In the present invention, measuring a value of A_2 or B_1 can be performed not only by absorbancy, but also by other known analytical methods instead.

As explained above, the present invention has advantages in that no measurement error can occur, due to use of coenzymes each having a different absorption in its reduced form, and in that the amounts of myo-inositol can also be assayed precisely and rapidly with even a small amount of specimen, due to combining enzymatic cycling reaction. In the present invention, use of heat stable myo-inositol dehydrogenase having a remaining activity over 95 % at 65°C is preferable.

Examples

The following examples illustrate the present invention but are not to be construed as limiting.

Example 1

Reagents:

40	mM	Glycine-NaOH buffer (pH 10.0)
2	mM	Thio-NAD (Sankyo Co.)
0.2	mM	reduced NAD (Oriental Yeast Co.)
150	U/ml	Myo-inositol dehydrogenase (Toyo Jozo Co. Bacillus sp. No. 3)

Procedure:

The above reagent mixture (1 ml) was put into cuvette and 20 µl of each of a range of concentrations of myo-inositol solution (0, 10, 20, 30, 40 and 50 µ M, respectively) was added thereto, with the reaction temperature at 37°C. After incubation commenced, a difference in absorbance at 400 nm at 2 mins. and 7 mins. was measured. The results are shown in Fig. 5, from which it can be seen that a linear relation between the amount of myo-inositol and the change in absorption was observed.

Example 2

Reagents:

40	mM	Glycine-NaOH buffer (pH 9.5)
2	mM	Thio-NAD (Sankyo Co.)
0.1	mM	reduced deamino NAD (Sigma Co.)
200	U/ml	Myo-inositol dehydrogenase (Toyo Jozo Co. *Bacillus* sp. No. 3)

Procedure:

The above reagent mixture (1 ml) was put into a cuvette and 50 μl of each of a range of concentrations of myo-inositol solution (0, 2, 4, 6, 8 and 10 μ M, respectively) was added thereto, then the mixture was incubated at 37°C for 60 minutes. Then the reaction was stopped by adding 0.5 % sodium dodecyl sulfate (1 ml). Absorbance at 400 nm was measured. The results are shown in Fig. 6, from which it can be seen good linear quantitative curve.

Example 3

Reagents:

50	mM	Glycine-NaOH buffer (pH 10.0)
0.2	mM	reduced NAD (Oriental yeast Co.)
4	mM	thio-NAD (Sankyo Co.)
250	U/ml	Myo-inositol dehydrogenase (Toyo Jozo Co. *Bacillus* sp. No. 3)
0.2	%	Triton X - 200

Procedure:

The above reagent mixture (1 ml) was put into cuvette and 20 μl of each of three different serum was added thereto, with the reaction temperature at 37°C. After incubation commenced, a difference in absorbance at 400 nm at 5 min. and 6 min. was measured.

50 μ M myo-inostiol solution (standard solution) and distilled water (reagent blank) were treated in the same manner as above and an amount of myo-inositol in serum samples was calculated from the difference in absorbance between the standard solution and samples.

In table hereinbelow, the results obtained from three different serum are shown.

	Difference Absobance (mAbs)	Myo-inositol concentration
Reagent blank	2	—
Standard solution	29	50 μM
Serum 1	25	42.6 μM
Serum 2	21	35.2 μM
Serum 3	31	53.7 μM

Example 4

Reagents:

40 mM Glycine-NaOH buffer (pH 10.0)
15 mM NADP (Oriental yeast Co.)
50 μM thio-NAD (Sankyo Co.)
0.4 M Ethanol
30 U/ml Alcohol dehydrogenase (Oriental yeast Co.)
250 U/ml Myo-inositol dehydrogenase (Toyo Jozo Co. Bacillus sp. No. 3)

Procedure:

The above reagent mixture (1 ml) was put into cuvettes and 50 μl of each of concnentrations of myo-inositol solution (0, 20, 40, 60, 80 and 100 μ M, respectively) was added thereto, with the reaction temperature at 37°C. After incubaion commenced, a difference in absorbancy at 340 nm at 3 mins and 8 mins. was measured. The results are shown in Fig. 7.

Example 5

Reagents:

50	mM	Phosphate buffer (pH 7.0)
0.25	mM	reduced NADP (Oriental yeast Co.)
50	μM	thio-NAD (Sankyo Co.)
5	mM	Dihydroxyacetone phosphate
10	U/ml	Glycerol-3-phosphate dehydrogenase (Boehringer Mannheim, rabbit muscle)
250	U/ml	Myo-inositol dehydrogenase (Toyo Jozo Co. Bacillus sp. No. 3)

Procedure:

The above reagent mixture (1 ml) was put into cuvettes and 50 μl of each of concnentrations of myo-inositol solution (0, 50, 100, 150, 200 and 250 μM, respectively) was added thereto, with the reaction temperature at 37°C. After incubaion commenced, a difference in absorbance at 340 nm at 3 mins. and 8 mins. was measured. The results are shown in Fig. 8.

Example 6

Culture Bacillus sp. No. 3:

Yeast extract (Kyokuto Seiyaku Co.)	2 %
Peptone (Kyokuto Seiyaku Co.)	2 %
K_2HPO_4 (Wako Pure Chem. Co.)	0.2 %
$CaCl_2$ (Wako Pure Chem. Co.)	0.02 %
$MgSO_4 \cdot 7H_2O$ (Wako Pure Chem. Co.)	0.05 %
Myo-inositol (Wako Pure Chem. Co.)	2 %
pH 7.3	

100 ml of a liquid medium comprising the above composition was sterilized in a 500 ml Erlenmeyer flask at 120°C for 20 mins. One loopful of Bacillus sp. No. 3 was inoculated into the medium and the medium was cultured at 50°C with stirring at 120 r.p.m. for 30 hours to obtain the cultured mass (85 ml)(enzyme activity: 1.2 U/ml). 20 l of a liquid medium comprising the above composition added with disfom CB 442 (Nihon Yushi Co.) 0.1 % was sterilized in a 30 l jar fermenter by heating. 85 ml of the pre-cultured seed culture obtained in the step above was inoculated therein and the mixture was cultured at 50°C, with aeration of 20 l/ml., inner pressure 0.4 kg/cm², and agitation at 150 r.p.m. for 24 hours to obtain the cultured mass (18.0 l)(enzyme activity: 1.8 U/ml).

Example 7

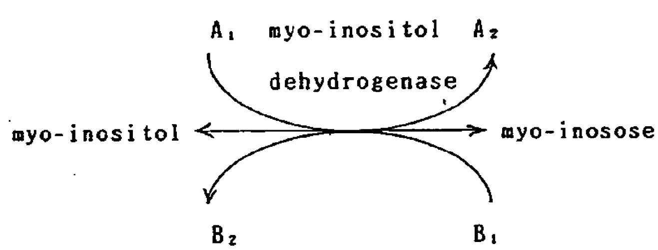
Purification of enzyme

Bacterial cells collected by centrifugation from the cultured broth obtained in Example 6 were suspended in 20 mM phosphate buffer (pH 7.5, 5 l) containing 0.1% lysozyme (Eizai Co.) and solubilized at 37°C for 1 hour; then the mixture was centrifuged to remove precipitate and to obtain a supernatant solution (4500 ml) (activity: 6 U/ml). Acetone (1.8 l) was added to the supernatant solution to separarte the precipitate, which was dissolved in 20 mM phosphate buffer to obtain crude extract (1 lit., 24.2 U/ml).

Ammonium sulfate (200 g) was added to the solution, Which was mixed well by stirring and then centrifuged to separate the precipitate. An additional 250 g ammonium sulfate was then added to the supernatant solution, and the solution was centrifuged to obtain a new precipitated. The new precipitate was dissolved in 20 mM phosphate buffer (pH 7.5) to obtain enzyme solution (500 ml, specific activity 36.3 U/ml), and the resultant solution was dialyzed over-night against 20 mM phosphate buffer (pH 7.5, 20 lit.). The dialyzed enzyme solution was charged on a column of DEAE-Sepharose CL-6B (Pharmacia Co.)(250 ml) which was bufferized with 20 mM phosphate buffer (pH 7.5), washed with 20 mM phosphate buffer containing 0.1 M KCl, (pH 7.5, 1 lit) and eluted with 20 mM phosphate buffer containing 0.3 M KCl (pH 7.5) to obtain an enzyme solution (350 ml, activity 35.2 U/ml). The enzyme solution was dialyzed over-night against 10 mM phosphate buffer (pH 7.0, 20 lit.) Bovine serum albumin (Sigma Co., 0.2 g) was dissolved in the thus-obtained enzyme solution, then the solution was lyophilized to obtain the lyophilized enzyme (1.1 g, 10.6 U/mg).

Claims

1. A method of assaying a specimen for myo-inositol which comprises reacting the specimen with:
   a) myo-inositol dehydrogenase using one of the thionictoinamide adenine dinucleotide phosphate group (thio-NADP group) or thionicotinamide adenine dinucleotide group (thio-NAD group) and one of the nicotinamide adenine dinucleotide phosphate group (NADP group) or nicotinamide adenine dinucleotide group (NAD group) as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,
   b) A_1 , and
   c) B_1 ;
   to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

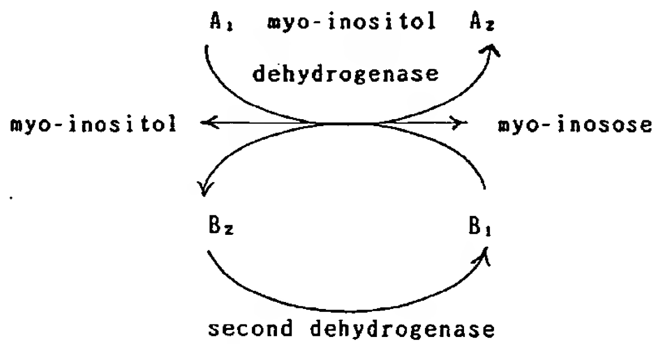
B_2 is an oxidized form of B_1; and

measuring the amount of A_2 or B_1 generated or consumed in the cycling reaction.

2. A method of assaying a specimen for myo-inositol which comprises reacting the specimen with:
   a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 ,
c) B_1 or/and B_2, and
d) a second dehydrogenase which does not act on myo-inositol and which catalyses a reaction forming B_1 from B_2 , and a substrate for the said second dehydrogenase;

to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

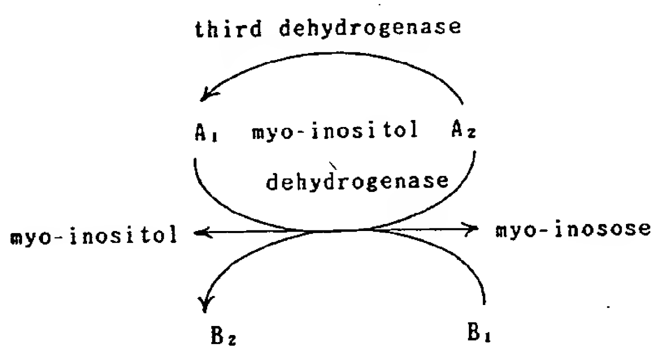
B_2 is an oxidized form of B_1;

the reaction from B_2 to B_1 being an enzymatic reaction which generates B_1 from B_2 by an action of the second dehydrogenase with a coenzyme; and

measuring the amount of A_2 generated in the cycling reaction.

3. A method of assaying a specimen for myo-inositol which comprises reacting the specimen with:
a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from Myo-inositol,
b) A_1 or/and A_2 ,
c) B_1 and
d) a third dehydrogenase which does not act on myo-inositol and which catalyses a reaction from A_2 to A_1 , and a substrate for the said third dehydrogenase;

to effect the cycling reaction:



wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

A_2 is a reduced form of A_1,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a NADP or NAD group compound, and

B_2 is an oxidized form of B_1;

the reaction from A_2 to A_1 being an enzymatic reaction which generates A_1 from A_2 by an action of the third dehydrogenase with a coenzyme; and

measuring the amount of B_1 consumed in the cycling reaction.

4. A method according to any one of the preceding claims wherein the thio-NADP group is thionicotinamide adenine dinucleotide phosphate (thio-NADP) or thionicotinamide hypoxanthine dinucleotide phosphate.

5. A method according to any one of claims 1 to 3 wherein the thio-NAD group is thionicotinamide adenine dinucleotide (thio-NAD) or thionicotinamide hypoxanthine dinucleotide.

6. A method according to any one of the preceding claims wherein the NADP group is nicotinamide adenine dinucleotide phosphate (NADP), acetylpyridine adenine dinucleotide phosphate (acetyl NADP) or nicotinamide hypoxanthine dinucleotide phosphate (deamino-NADP).

7. A method according to any one of claims 1 to 5 wherein the NAD group is nicotinamide adenine dinucleotide (NAD), acetylpyridine adenine dinucleotide phosphate (acetyl NAD), or nicotinamide hypoxanthine dinucleotide phosphate (deamino-NAD).

8. A reagent composition suitable for assaying myo-inositol which comprises:

a) myo-inositol dehydrogenase using one of the thionicotinamide adenine dinucleotide phosphate (thio-NADP group) or thionicotinamide adenine dinucleotide group (thio-NAD group) and one of the nicotinamide adenine dinucleotide phosphate group (NADP group) or nicotinamide adenine dinucleotide group (NAD group) as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 and

c) B_1;

wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound.

9. A reagent composition suitable for assaying myo-inositol which comprises:

a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 ,

c) B_1 or/and B_2, and

d) a second dehydrogenase which does not act on myo-inositol and which catalyzes a reaction forming B_1 from B_2, and a substrate for the said second dehydrogenase,

wherein:

A_1 is a thio-NADP, thio-NAD, NADP or NAD group compound,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

B_2 is an oxidized form of B_1.

10. A reagent composition suitable for assaying myo-inositol which comprises:

a) myo-inositol dehydrogenase using one of the thio-NADP group or thio-NAD group and one of the NADP group or NAD group as coenzymes, which myo-inositol dehydrogenase catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A_1 or/and A_2

c) B_1 and

d) a third dehydrogenase which does not act on myo-inositol and which catalyzes a reaction from A_2 to A_1, and a substrate for the said third dehydrogenase;

wherein:

A_1 is thio-NADP, thio-NAD, NADP or NAD group compound,

B_1 is a reduced NADP or reduced NAD group compound when A_1 is a thio-NADP or thio-NAD group compound or B_1 is a reduced thio-NADP or reduced thio-NAD group compound when A_1 is a NADP or NAD group compound, and

A_2 is an oxidized form of A_1 .

11. A myo-inositol dehydrogenase which has substrate specificity for myo-inositol and which catalyses the reaction:

$$\text{myo-inositol} + \text{NAD} \rightleftarrows \text{myo-inosose} + \text{reduced NADH}$$

12. A myo-inositol dehydrogenase according to claim 11 which has the following characteristics:

(1) molcular weight : 130.000 ± 15,000 (gelfiltration method by TSK gel G 3000 SW)
(2) iso-electric point: pH 4.5 ± 0,5
(3) Km-value :
Km value for myo-inositol ; 0,64 mM
Km value for NAD ; 0.004 mM
(4) optimum pH : approx. pH 9.5
(5) pH-stability: more than 80% of remaining activity at a pH of from 6.5 to 9.0

13. A process for the production of a myo-inositol dehydrogenase which comprises culturing a myo-inositol dehydrogenase producing microorganism belonging to genus Bacillus, and isolating myo-inositol dehydrogenase from the cultured mass.

14. A process according to claim 13 wherein the myo-inositol dehydrogenase producing microorganism is Bacillus sp. No. 3 (FERM BP-3013) or a mutant thereof which is capable of producing the myo-inositol dehydrogenase.

15. Bacillus sp. No. 3 (FERM BP-3013) or a mutant thereof which is capable of producing a myo-inositol dehydrogenase.

16. A culture of Bacillus sp. No. 3 (FERM BP-3013), or a mutant thereof which is capable of producing a myo-inositol dehydrogenase, in a culture medium which comprises a source of assimilable carbon, a source of assimilable nitrogen and mineral salts and which is free of other microorganisms.

FIG. 1

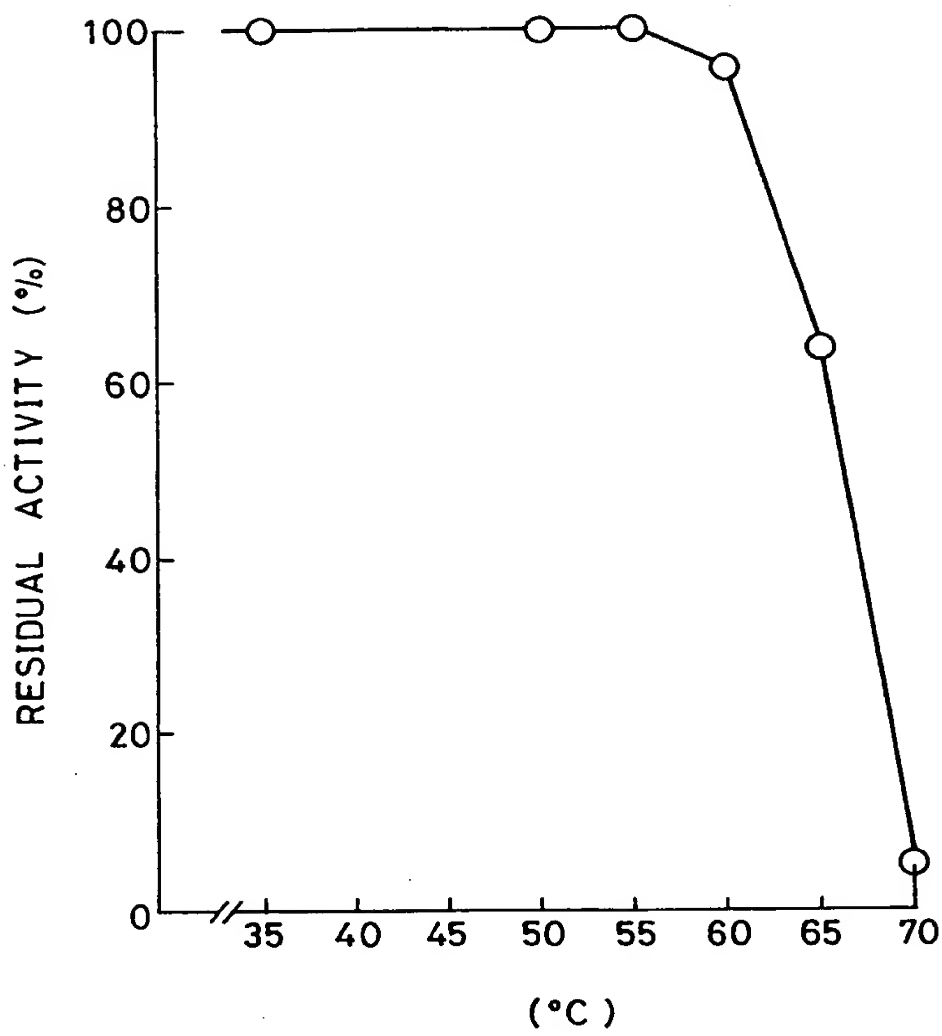


FIG. 2

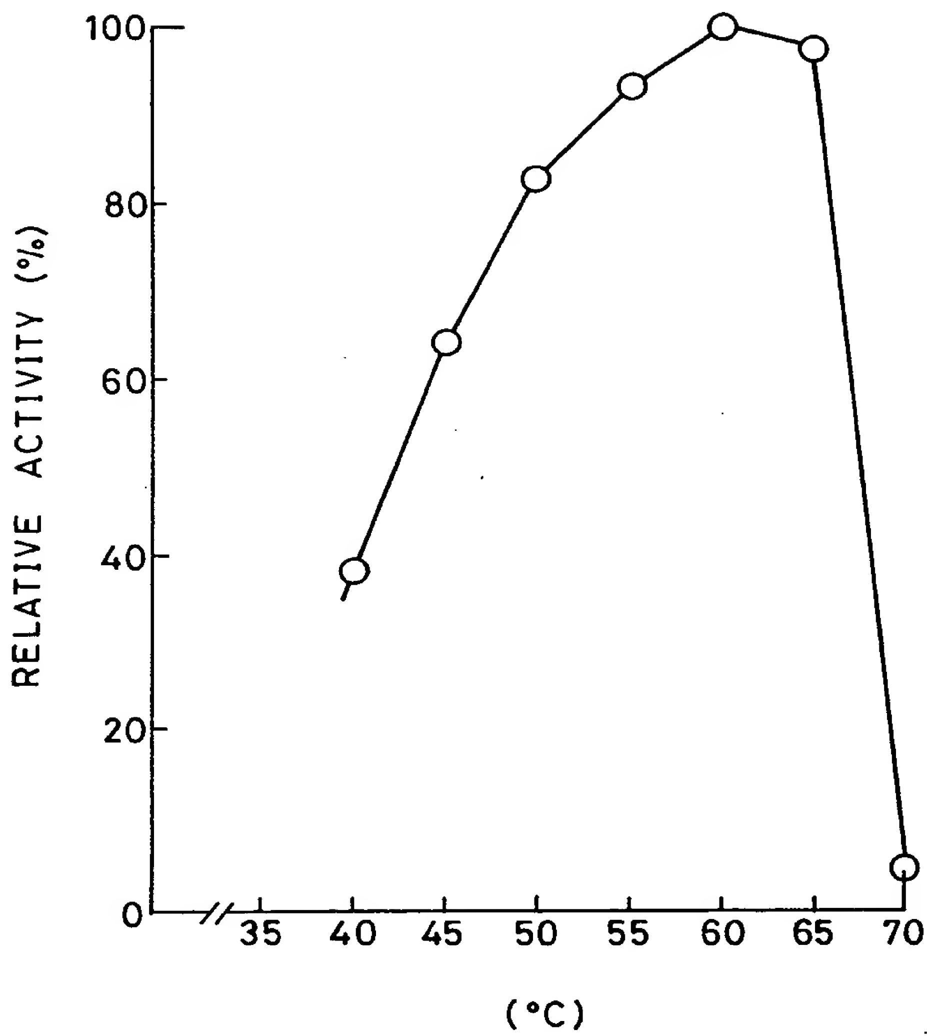


FIG. 3

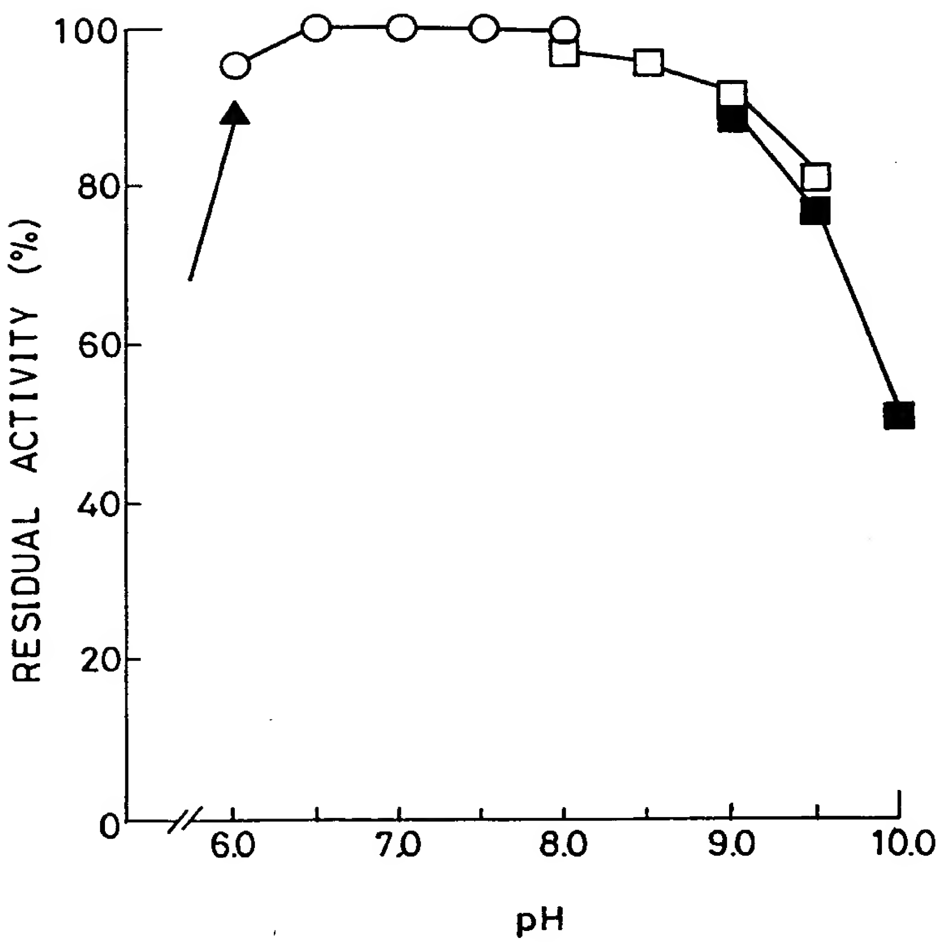


FIG. 4

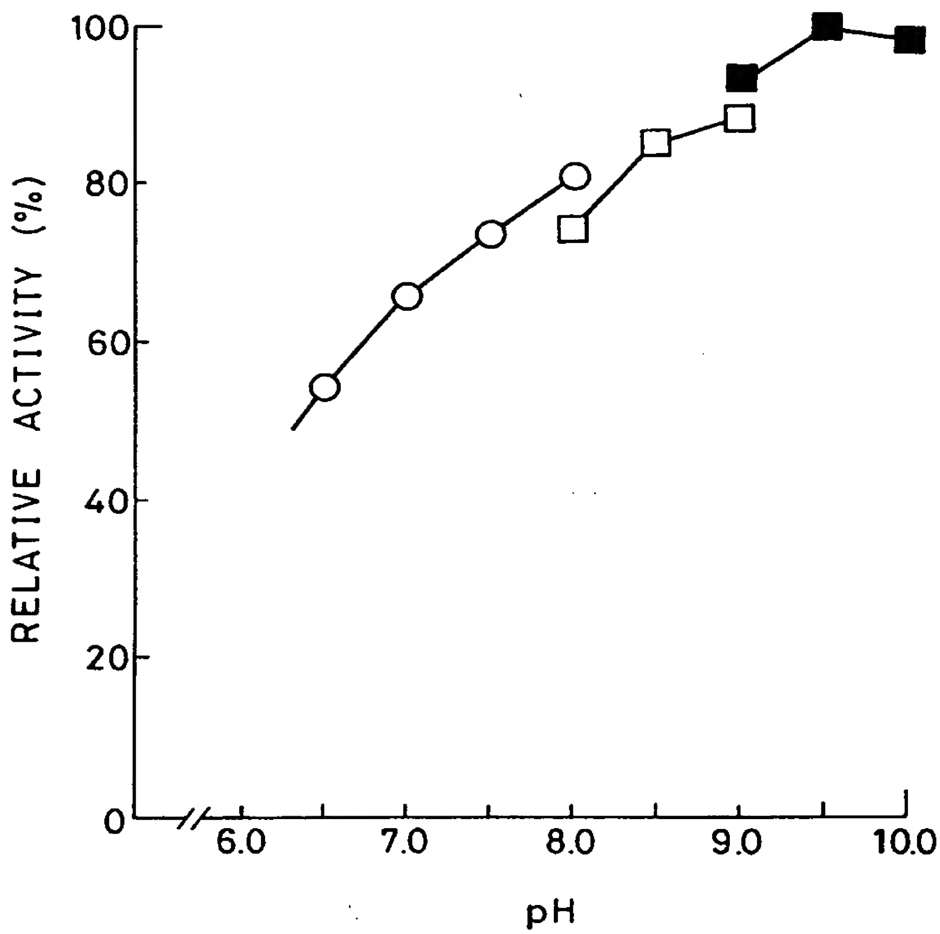


FIG. 5

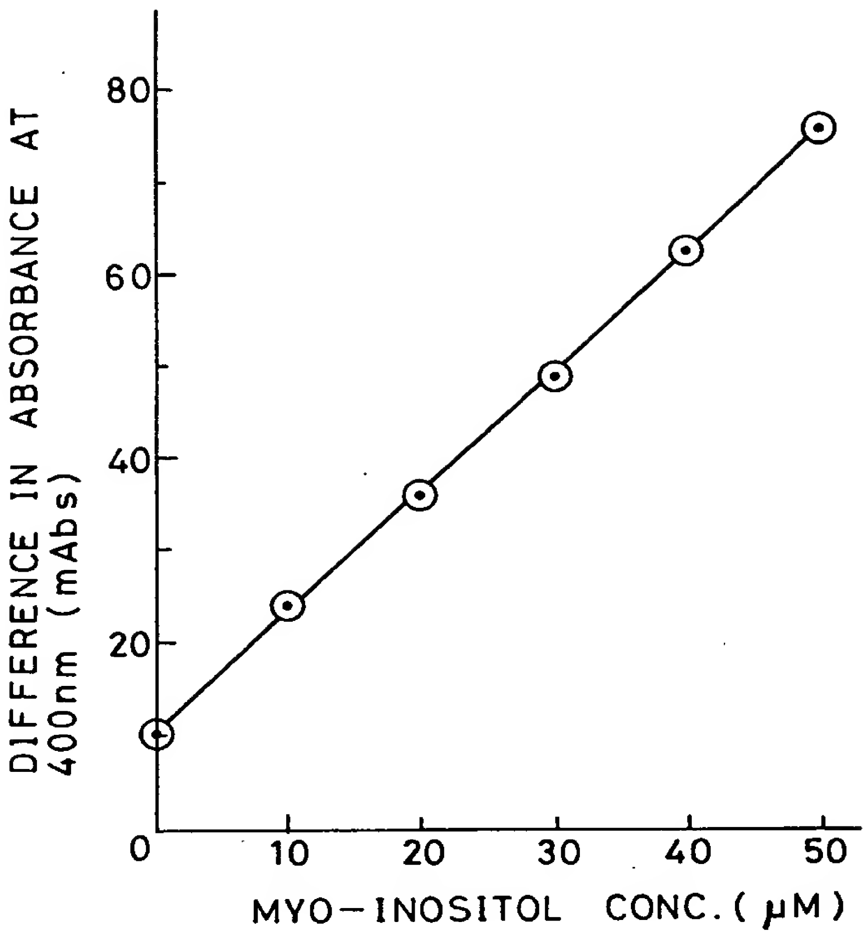

DIFFERENCE IN ABSORBANCE AT 400nm (mAbs)
80
60
40
20
0
10
20
30
40
50
MYO-INOSITOL CONC. (μM)

FIG. 6

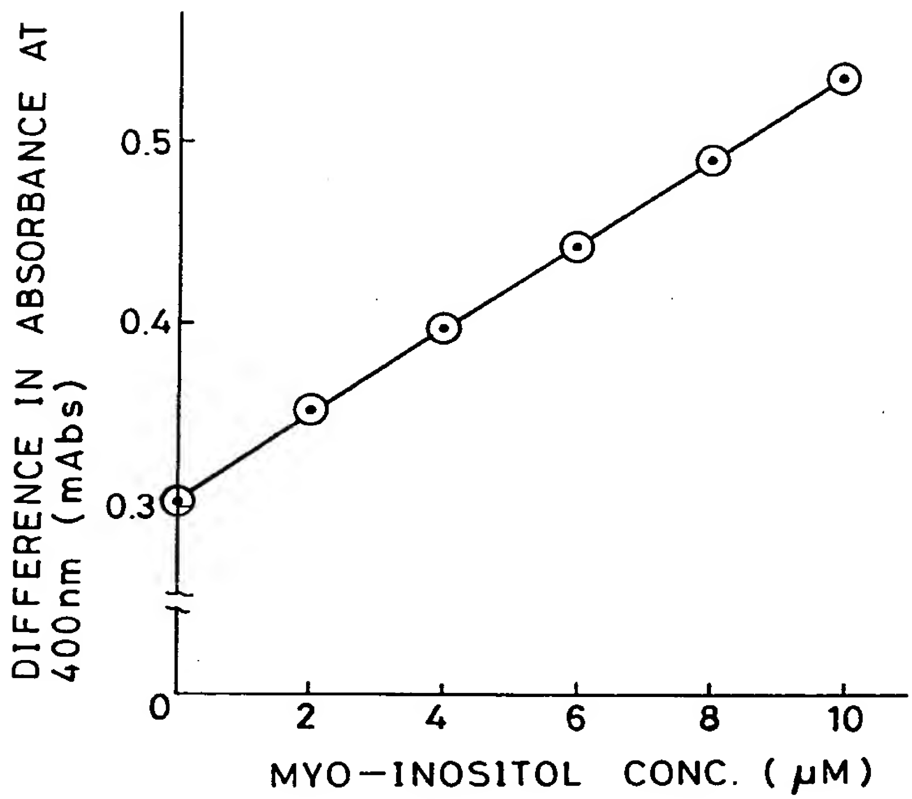

DIFFERENCE IN ABSORBANCE AT 400nm (mAbs)
0.5
0.4
0.3
0
2
4
6
8
10
MYO-INOSITOL CONC. (μM)

FIG. 7

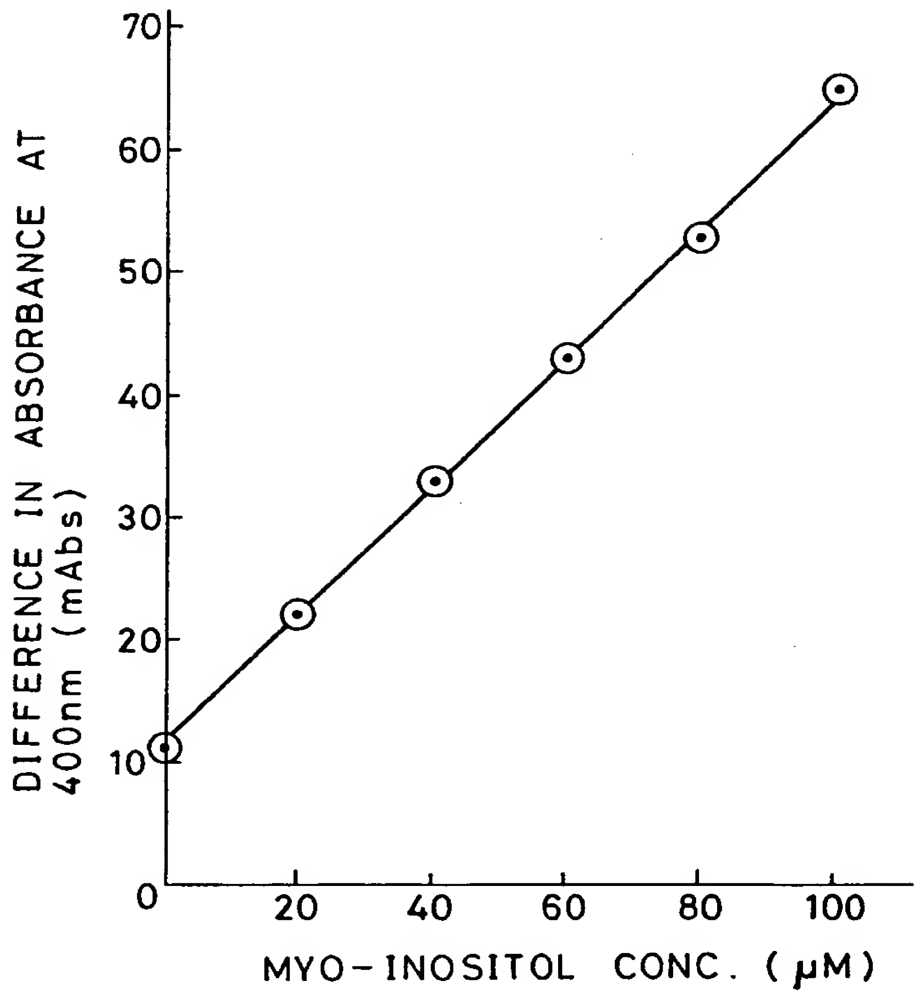
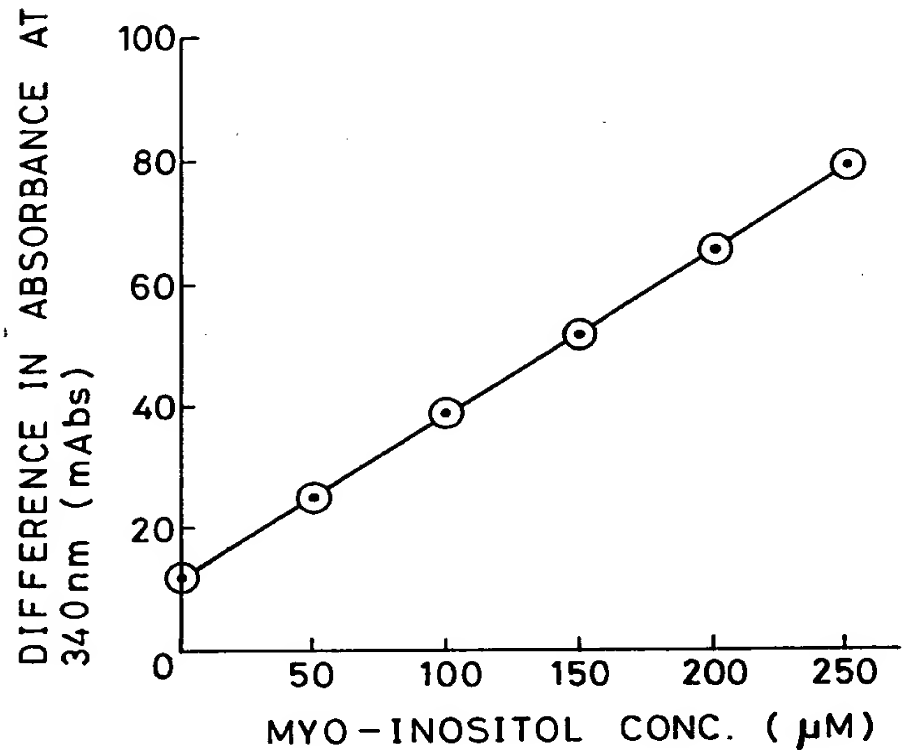

DIFFERENCE IN ABSORBANCE AT 400nm (mAbs)
0
10
20
30
40
50
60
70
20
40
60
80
100
MYO-INOSITOL CONC. (μM)

FIG. 8


DIFFERENCE IN ABSORBANCE AT 340nm (mAbs)
100
80
60
40
20
0
50
100
150
200
250
MYO-INOSITOL CONC. (μM)